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1 **Title**

2 Assessment of the interplay between scaffold geometry, induced shear stresses and cell
3 proliferation within a packed bed perfusion bioreactor.

4

5 **Running tittle**

6 Assessment of optimal shear stress within a bioreactor

7

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27 **Abstract**

28 By favouring cell proliferation and differentiation, perfusion bioreactors proved efficient at
29 optimizing cell culture. The aim of this study was to quantify cell proliferation within a
30 perfusion bioreactor and correlate it to the wall shear stress distribution by combining 3-D
31 imaging and computational fluid dynamics simulations.

32 NIH-3T3 fibroblasts were cultured onto a scaffold model made of impermeable polyacetal
33 spheres or Polydimethylsiloxane cubes. After 1, 2 and 3 weeks of culture, constructs were
34 analysed by micro-computed tomography (μ CT) and quantification of cell proliferation was
35 assessed. After 3 weeks, the volume of cells was found four times higher in the stacking of
36 spheres than in the stacking of cube.

37 3D- μ CT reconstruction of bioreactors was used as input for the numerical simulations. Using
38 a lattice-Boltzmann method, we simulated the fluid flow within the bioreactors. We retrieved
39 the wall shear stress (WSS) distribution (PDF) on the scaffolds surface at the beginning of
40 cultivation and correlated this distribution to the local presence of cells after three weeks of
41 cultivation. We found that the WSS distributions strongly differ between spheres and cubes
42 even if the porosity and the specific wetted area of the stackings were very similar. The PDF
43 is narrower and the mean WSS is lower for cubes (11 mPa) than for spheres (20 mPa). For the
44 stacking of spheres, the relative occupancy of the surface sites by cells is maximal when WSS
45 is greater than 20 mPa. For cubes, the relative occupancy is maximal when the WSS is lower
46 than 10 mPa. The discrepancies between spheres and cubes are attributed to the more
47 numerous sites in stacking of spheres that may induce 3-D (multi-layered) proliferation.

48

49

50 **Introduction**

51 Perfusion bioreactors are widely used in musculoskeletal tissue engineering ¹⁻⁵. Bone cells are
52 sensitive to wall shear stress (WSS) ⁶⁻¹⁵, influencing cell fate through mechanotransduction ¹⁶⁻
53 ²⁰, resulting in an enhancement of cell proliferation and/or differentiation ^{1,6,7,21-24}. The
54 capacity of a perfusion bioreactor to lead to bone tissue has been demonstrated using animal
55 models ^{2,25}. Two strategies can be implemented to investigate WSS influence at the single-cell
56 scale. The first involves developing dedicated microsystems to study the phenomena and then
57 transferring the results to the bioreactor scale. The second requires examining the bioreactor
58 scale directly and using the appropriate means to collect information at the single-cell scale.
59 The heterogeneity of the local conditions encountered within a bioreactor limits *a priori* the
60 interpretation of the results. However, if the heterogeneity of local conditions is known (using
61 numerical simulation), this understanding grants access to an information bank that, can then
62 be statistically analysed.

63 Today, histology is still the standard method for investigating engineered constructs.
64 However, this method provides only a partial visualization of the construct. Many researches
65 have attempted to use micro-computed tomography (μ CT) to determine porosity or other
66 characteristics within the scaffold as an alternative to histology. This technique allows for
67 visualization and quantification of dense structures, and includes the possibility of
68 investigating the influence of scaffold features, such as the internal pore architecture with
69 regard to the growth of cells within the scaffold ²⁶⁻³⁰. Authors have previously reported that
70 μ CT can be used to image and segment microporous scaffolds seeded with mesenchymal stem
71 cells (MSCs) and cultured to produce soft tissue and mineralized tissue in a bioreactor with a
72 high degree of accuracy ^{28,30}. Furthermore, image processing is a key factor in establishing a
73 relevant method for separating different phases (porosity, cells and biomaterials) from μ CT
74 images ³¹. Authors managed to segment images using an in-house segmentation algorithm

75 ^{28,29}. 3D- μ CT reconstructions can be used to simulate flow and model the relationship
76 between cell proliferation and mechanical quantities, including shear stress. Thus, these
77 reconstructions help determine which solicitations that seem to be the most favourable for cell
78 expansion. Authors report a proof-of-principle, demonstrating that it is possible to model the
79 real geometrical and hydro-dynamical microenvironment within a scaffold and the presence
80 of cells in micro-pores. Various modelling approaches carried out within the last decade are
81 reported in the literature ³²⁻³⁵. To evaluate the localized fluid shear stress distributions
82 throughout the culture period from micro-tomography images, giving an indication of the
83 effects of tissue growth on the flow-induced stress field, the authors deployed fluid dynamic
84 simulations using a custom in-house lattice Boltzmann (LBM) program ³⁰. LBM easily
85 handles complex geometries such as porous media. After binarization, the 3D- μ CT image of a
86 porous medium can be used directly by the LBM code (just by locating the nodes of the lattice
87 at the centre of the voxels).

88 This paper describes a method to identify the WSS optimal range for cell proliferation within
89 constructs cultured in a packed bed bioreactor. This method is based on a combined analysis
90 of micro-tomography images and Computational fluid dynamics (CFD) simulations. We
91 present an illustration based on a bioreactor having demonstrated its ability to produce bone
92 constructs resulting in bone formation in a large animal ². Because our aim was not to produce
93 a bone construct, but rather to establish and validate a method, we chose to use fibroblast cells
94 because of their known mechanosensitivity ³⁶, cultured onto biocompatible ³⁷ impermeable
95 Polyacetal (POM) spheres or Polydimethylsiloxane (PDMS) cubes within the bioreactor.
96 POM spheres have several excellent qualities, including wear resistance, low moisture
97 absorption, dimensional stability, good fatigue strength and thermal stability ³⁸⁻⁴⁰. After 1, 2
98 or 3 weeks of culture, samples were analysed using a 3D imaging system based on μ CT. We
99 made the cells dense to X-rays using osmium-tetroxide staining. By combining this imaging

100 method with image processing and analysis, we quantified the cell proliferation according
101 time. We used the μ CT 3D reconstruction of our bioreactors as input for the CFD simulations.
102 Fluid flow was simulated within the packed bed perfusion bioreactors using a LBM. We
103 analysed the wall shear stress distribution provided by the numerical simulation together with
104 the scaffold surface coverage with cells to identify the wall shear stress values that were most
105 favourable to proliferation.

106

107 **Materials and methods**

108 **Cell line**

109 Murine fibroblasts NIH/3T3 (ATCC) were cultured in DMEM Glutamax (Gibco)
110 supplemented with 10% Foetal Calf Serum (Pan-Biotech) in standard conditions (37 °C, 5%
111 CO₂, 100% humidity).

112 **Perfusion bioreactor system**

113 A pre-approved, custom-made perfusion bioreactor ² was used in the present study. The
114 perfusion bioreactor was connected to a reservoir containing the culture medium in a closed
115 loop arrangement of platinum-cured silicone tubing (Masterflex). The perfusion bioreactor
116 was located within an incubator set at 37°C. The peristaltic pump (Masterflex) was set up to
117 deliver a flow rate Q of 10 mL/min. This design allowed the medium to flow from the
118 reservoir to an entry chamber located at the bottom of the perfusion bioreactor, preventing
119 fluid flow disturbances and maintaining uniform flow conditions. To maintain sample
120 integrity when extracting the sample from the bioreactor, we placed inside the perfusion
121 chamber a Polypropylene tube (inner diameter 12 mm, 50 mm height) that was designed to fit
122 the chamber's internal diameter. This ensured that the media would flow within the tube. A
123 grid (with pores of 1 mm in diameter) separated the entry from the perfusion bioreactor
124 chamber (where scaffolds were placed during experiments).

125 **3D spheres or cubes-based constructs**

126 We used nonporous biocompatible 3D materials in our experiments. Polyacetal spheres (2mm
127 Ø) (Marteau & Lemarié) or Polydimethylsiloxane cubes (3mm*3mm*3mm) (Sylgard®,
128 custom-made mold) acted as a scaffold (packed bed configuration). Materials 'dimension

129 were chosen according to previous study ² to comply with a rough porosity of 50% and an
130 estimated shear stress up to 100mPa.

131 **Seeding and culturing cells in perfusion bioreactor**

132 Tubes (15mL) were filled with 300 POM spheres or 60 PDMS cubes. Prior to cell seeding,
133 scaffolds were treated with fibronectin (10 mg/mL, 1 hour, 37°C, Roche). 75% confluent 3T3
134 cells (10⁶ cells/mL) were then seeded onto the scaffold. Scaffolds were let overnight in a 5%
135 CO₂ incubator. To assess cell number, control samples (n=2) were performed. Cell number
136 was assessed after trypsinization (1 x 10 min) using Malassez cell.

137 After 24 hours, seeded scaffolds were placed within the perfusion bioreactor and the medium
138 was allowed to flow at a continuous rate of $Q = 10$ mL/min. Once a week the medium
139 (200mL) was renewed. Culture under perfusion was carried out for 1, 2 and 3 weeks. For each
140 time point, the results reported are average values of 3 independent experiments.

141 **Sample preparation for X-ray micro-tomography.**

142 At the indicated time points, constructs were fixed overnight (4% PFA in PBS, Sigma-
143 Aldrich), washed with PBS and stained overnight with 1% osmium-tetroxide (OsO₄ in PBS,
144 Alpha Aesar) to increase cellular contrast and makes them visible by μ CT. After staining,
145 samples were dehydrated by gradually increasing ethanol bath at 25, 50, 70, 90, 95, and
146 100%. A final bath of hexamethyldisilazane (Sigma) was performed to completely dry out the
147 sample. HMDS drying is a good alternative to critical point drying in the preparation of the
148 samples. This method is time and cost-effective ⁴¹, as well as optimal in order to dehydrate
149 cells (low distortion) ⁴².

150 **X-ray computed micro-tomography image acquisition**

151 A μ CT system X-view X 50-CT (North Star Imaging), was used to non-destructively image
152 and quantify biomass production for each sample. μ CT experiments were performed with a
153 target of Molybdenum producing a polychromatic beam used for low absorption samples.
154 Acquisition setup was the following: the accelerating voltage was set to 50 kV with a tube
155 current of 400 μ A. 1,200 projections were obtained from each sample over 360° (0.3°
156 intervals) with an exposure time of 3 seconds per projection (averaging of 20 images). The
157 transmitted beam was detected by a CMOS image sensor (3888*3072 pixels) coupled to a CsI
158 scintillator. Each sample was scanned with a pixel size of 17 μ m*17 μ m, yielding a field of
159 view of about 3 cm. The average scanning time of each specimen was 90 minutes.

160 **Image reconstruction, visualization and analysis**

161 efX-ct software (North Star Imaging) was used to reconstruct the 2D absorption projection
162 images, generating a set of cross-sectional slices of the scaffold. Greyscale images were then
163 exported as 16 bit images for quantitative purposes. We used ImageJ software 1.48V to
164 process and quantify X-CT images. The region of interest corresponded to the volume
165 delimited by the inner wall of the bioreactor. For each bioreactor, we measured the construct
166 porosity and the volume of the cellular phase. 16 bits RAW images were segmented using a
167 thresholding method to separate scaffolds from the cells. Threshold values were retrieved
168 from the mean of all samples (either POM spheres or PDMS cubes). Additional image
169 processing was performed. First, a linear calibration and a Gaussian Blur mask were
170 performed on the constructs and background respectively to homogenize the grey scale value.
171 Then, a region of interest was determined, corresponding to the volume delimited by the inner
172 wall of the bioreactor. Finally, cells and constructs were extracted from images using
173 ImageJParticleAnalyzer3D plugin.

174 **Fluid Flow Simulations**

175 Following Porter⁴³, we computed the fluid flow within the perfusion bioreactor and the shear
176 stress distribution at the solid-fluid interface using an in-house lattice-Boltzmann code.
177 Lattice-Boltzmann methods are particularly well-suited to investigate fluid flow at low
178 Reynolds number in complex geometries.

179 The culture medium is assumed to be incompressible and Newtonian. Its dynamic viscosity at
180 37°C is estimated at $\mu = 10^{-3}$ Pa.s⁴⁴. The natural convection within the bioreactor is
181 neglected compared to the forced convection due to the perfusion since the Richardson
182 number (estimated with the temperature accuracy of the incubator, *i.e.* about 0.1°C, and the
183 materials size) is significantly lower than 1 ($Ri \approx 0.1$). The flow can therefore be considered
184 as isothermal. The particle Reynolds number (estimated with the bead or cube size) is equal to
185 $Re \approx 3 - 4.5$. According to Bağcı⁴⁵, the perfusion flow within the granular bed formed by the
186 POM beads or the PDMS cubes is thus in the Darcy regime ($Re \leq 22$).

187 The fluid flow within the bioreactor can be described by the well-known continuity and
188 Navier-Stokes equations. We simulated these equations using the multiple-relaxation-time
189 lattice-Boltzmann Method (MRT LBM) proposed by d’Humières⁴⁶ with a cubic lattice in 3
190 dimensions and 19 velocities, *i.e.* $d3q19$ (see Supplementary material, Fig. S1). We remind
191 that a LBM is built as follows: first of all, the d -dimensional space is mapped on a regular
192 lattice. Two types of lattice nodes are distinguished: the first type corresponds to the solid
193 phase, the second to the space left to the fluid. A population of fictitious particles is allocated
194 to each node of the space filled by the fluid. The velocity distribution of the population is
195 discrete. q particle velocity vectors are defined: one velocity vector is the null vector and the
196 others are associated with the links between a node and its $(q - 1)$ neighbors. At each time
197 increment, the population dynamics is divided into two steps: propagation of the fictitious
198 particles from node to node according to their velocity, then relaxation of the particle

199 populations toward equilibrium. LBM is formulated to recover the Navier–Stokes equations
 200 as the macroscopic behavior of the fictitious particles.

201 The evolution equation of the particle population f_i with velocity \vec{c}_i located at the position \vec{r}
 202 at time t typically reads (in lattice units):

$$203 \quad f_i(\vec{r} + \vec{c}_i, t + 1) = f_i(\vec{r}, t) + \mathcal{C}_i \left(\left(f_j(\vec{r}, t) \right)_{j=0,q} \right) \quad (1)$$

204 where \mathcal{C}_i is the collision operator. We used a collision operator with two relaxation
 205 parameters⁴⁷. The first one, ω_1 , depends on the fluid kinematic viscosity ν :

$$206 \quad \nu = c_s^2 \left(\frac{1}{\omega_1} - \frac{1}{2} \right) \frac{\Delta x^2}{\Delta t} \quad (2)$$

207 with c_s the intrinsic speed of sound of the LBM ($c_s^2 = 1/3$ in lattice units), Δx the lattice
 208 spacing (in real units) and Δt the time step (in real units). The second one, ω_2 , is fixed
 209 through the so-called “magic number combination” in order to minimize the spatial error
 210 associated with the bounce-back wall location⁴⁸:

$$211 \quad \omega_2 = 8 \frac{2 - \omega_1}{8 - \omega_1} \quad (3)$$

212 The hydrodynamic quantities we are interested in, are the moments of the velocity distribution
 213 $(f_i)_{i=0,q}$. The fluid pressure P (in real units) is related to the zeroth order velocity moment:

$$214 \quad P = \rho \left(\frac{\Delta x}{\Delta t} \right)^2 c_s^2 \sum_{i=0}^q f_i \quad (4)$$

215 with ρ the fluid density (in real units). The fluid velocity \vec{v} (in real units) is the first order
 216 velocity moment:

217
$$\vec{v} = \frac{\Delta x}{\Delta t} \sum_{i=0}^q f_i \vec{c}_i \quad (5)$$

218 The lattice-spacing is fixed to 51 μm , *i.e.* three times the resolution of the μCT scans, to
219 reduce the computational effort. The nodes of the lattice are located at the centre of $(51 \mu\text{m})^3$
220 voxels. Since we simulated the fluid flow in a whole bioreactor chamber, the computational
221 domain (see Supplementary material, Fig. S2) comprises about 270 x 270 x 540 nodes.

222 The time step Δt is selected such as the compressibility effects in the LBM remain negligible.
223 This constraint reads:

224
$$\frac{v_{max}}{(\Delta x / \Delta t)} \lesssim 0.1 c_s \quad (6)$$

225 where v_{max} is the maximum velocity reached by the fluid in the domain.

226 A flat and constant velocity profile is set at the inlet of the chamber, *i.e.* the component of the
227 fluid velocity parallel to bioreactor axis is equal to $v_s = 1.47 \text{ mm.s}^{-1}$. v_s is the superficial
228 velocity, *i.e.* the ratio of the perfusion flow rate Q to the tube cross-section. A zero velocity
229 gradient is assumed at the outlet⁴⁹. The complex solid boundaries (associated with the
230 bioreactor walls and the spheres or cubes forming the granular packed bed) are approximated
231 by a staircase shape (in accordance with the voxel representation provided by the μCT scans).
232 A no-slip (zero velocity) condition is assumed on the solid surfaces. This assumption is
233 relevant because the spheres and the cubes are non-porous. The no-slip condition is modelled
234 by the so-called bounce-back rule, in which a fictitious particle incident on the boundary
235 reverses its direction. In this way, the fictitious particles are kept out of the obstacles and the
236 null velocity condition is satisfied at the solid surface. The solid surface is located halfway
237 between the “fluid node” and the “solid node” forming the obstacle.

238 We ran LBM simulations until fluid flow reached steady state.

239

240 **Wall shear stress computation**

241 To determine the WSS that the fluid exerted, the viscous stress tensor $\bar{\sigma}$ and the wall normal
242 vector \vec{n} are required. The modulus of the wall shear stress τ is indeed given by:

$$243 \quad \tau = |\bar{\sigma} \cdot \vec{n} - ((\bar{\sigma} \cdot \vec{n}) \cdot \vec{n}) \vec{n}| \quad (7)$$

244 The viscous stress tensor can be calculated at each neighbour node of a solid surface from the
245 LBM velocity distribution function at that node. The nodes (and associated voxels)
246 neighbouring a solid surface (spheres, cubes or bioreactor lateral inner walls) are further
247 referred to as scaffold surface sites. The generic term $\sigma_{\alpha\beta}$ of the viscous stress tensor (in real
248 units) is given by:

$$249 \quad \sigma_{\alpha\beta} = \left(\frac{\omega_1}{2} - 1\right) \left(\rho \left(\frac{\Delta x}{\Delta t}\right)^2 \left(\sum_{i=0}^q f_i c_{i\alpha} c_{i\beta} \right) - v_\alpha v_\beta - P \delta_{\alpha\beta} \right) \quad (8)$$

250 where $c_{i\alpha}$ is the α -component ($\alpha = x, y$ or z) of the particle velocity \vec{c}_i , v_α is the α -
251 component of the fluid velocity \vec{v} and $\delta_{\alpha\beta}$ is the Kronecker symbol.

252 The determination of the boundary normal vector is not as straightforward, due to the
253 staircase shape of the solid boundary. We implemented a technique proposed by Stahl ⁵⁰ to
254 detect locally the wall normal direction. This method relies on the fluid flow properties near
255 the solid boundary and not on the boundary geometry itself, as in the marching cubes
256 algorithm ⁵¹. We refer the interested reader to the original article of Stahl et al. ⁵⁰ if he wishes
257 further information.

258 We computed the probability density function (PDF) of the wall shear stress modulus as
259 follows: the wall shear stress range is divided into bins of equal size, *i.e.* $\Delta\tau = 1$ mPa. The

260 value of the PDF in the i -th bin is equal to $N(i)/(\Delta\tau \sum_j N(j))$ where $N(i)$ is the number of
261 scaffold surface sites with WSS falling into the interval $[(i - 1)\Delta\tau ; i\Delta\tau]$. We also defined a
262 conditional PDF as the PDF of the WSS conditioned to the local presence of cells at a given
263 cultivation time. The value of the conditional PDF in the i -th bin is equal to
264 $N_c(i)/(\Delta\tau \sum_j N_c(j))$ where $N_c(i)$ is the number of scaffold surface sites covered by cells at
265 that time with WSS falling into the interval $[(i - 1)\Delta\tau ; i\Delta\tau]$.

266 **Statistical Analysis**

267 Experiments were carried out three times independently. Analyses were performed using
268 GraphPad Prism software (GraphPad).

269

270 **Results**

271 **Cell visualization by μ CT**

272 Osmium tetroxide allowed us to increase the density of the cell fraction and, in combination
273 with biomaterials chosen for their low X-ray attenuation properties (polyacetal, $d=1,41\text{g/cm}^3$;
274 PDMS, $d=1,1\text{g/cm}^3$), we were able to visualize cells with a high signal to background ratio
275 (Fig. 1). We optimized this approach to facilitate the quantification procedure based on
276 threshold method for converting the grey-scaled image into a binary one. Thus, it was
277 possible for us to separate the scaffold from cells.

278 **Quantification, spatial distribution and dynamic of cell proliferation within the** 279 **construct**

280 We prepared the granular packed bed by stacking POM spheres or PDMS cubes. We then
281 used this as a scaffold to investigate cell proliferation under a steady flow rate (10 mL/min).
282 We investigated cell proliferation over 3 weeks by X-ray μ CT visualization. During the
283 experiment, we were able to visualize cell proliferation over the entire volume of the
284 bioreactor (3 cm^3) (Fig. 2). Over the 3 weeks of culture, we quantified the total cellular
285 volume and simultaneously evaluated the local cell proliferation with regards to scaffold
286 geometry (Figure 2-B, 2-D). Quantification of the cellular volume revealed a considerable
287 increase from $3.6\text{ mm}^3 \pm 1.9$ at week 1 to $60.7\text{ mm}^3 \pm 24.4$ at week 3 for spheres and an
288 increase from $0.82\text{ mm}^3 \pm 0.13$ at week 1 to $15.75\text{ mm}^3 \pm 1.20$ at week 3 for cubes. Knowing
289 the volume of the cell phase for each culture time and the average volume of a fibroblast NIH-
290 3T3 ($\vartheta = 1540\text{ }\mu\text{m}^3/\text{fibroblast}$)⁵², it is possible to estimate the cell number. To validate this
291 hypothesis, additional experiments were carried out with spheres. After 1 week, the cell phase
292 volume was $3.6\pm 1.9\text{ mm}^3$ which represents 2.2 ± 1.0 million cells. Then, this data can be
293 compared to the real number of cells obtained by cell count. These data are in good agreement

294 with the cell counts carried out after one week of bioreactor culture on independent samples
295 (2.5 million cells).

296 Interestingly, for cubes, while homogeneous increase in area colonized by cells was observed
297 during the first 2 weeks along the longitudinal axis of the bioreactor, local variations were
298 noticed at week 3 (Fig. 3). These local variations were observed with a periodic recurrence of
299 3 mm, which corresponds to the size of the PDMS cube (Fig. 3-A), and maxima were located
300 in between the horizontal cube/cubes contact zone. Local variations of the cellular volume
301 were also observed for spheres (Fig. 3-B), although to a lesser extent, with a periodic
302 recurrence of 2 mm, which corresponds to the diameter of the spheres.

303 Analysis of volume distribution within the bioreactors allowed us to distinguish 3 classes of
304 cell aggregates, namely Small aggregates (with a volume below 1000 voxels; <2,500
305 fibroblasts), Medium aggregates (volume comprised between 1000 and 10,000 voxels;
306 between 2,500 and 25,000 fibroblasts) and Large aggregates (volume superior to 10,000
307 voxels; > 25,000 fibroblasts). Such distinctions allowed us to describe the proliferative
308 mechanism with regards to volume expansion observed within the constructs. We further
309 analyzed the volume and the number of cell aggregates within the bioreactor to better
310 understand the dynamics of cell proliferation over time-course of the experiment (Fig. 4).
311 When cultured on PDMS cubes under steady flow rate, we noticed that after 1, 2 or 3 weeks
312 of culture, Small aggregate were the most numerous within the bioreactor (Fig. 4-A).
313 However, the cellular volume they represent was constantly decreasing over the time of
314 culture. After 1 week of culture, they represented 55% of the cellular volume while
315 decreasing to 23.2% after 2 weeks and represented only 7.6% at week 3. Interestingly, we
316 observed the inverse phenomenon for large aggregates. At week 1 only 1 large aggregate was
317 detected, representing itself 14.54% of the cellular biomass at this time point. At week 2, 23
318 Large aggregates were detected representing a volume of 29.8% of the total cellular volume

319 and finally, at week 3 Large aggregates (n=95) accounted for 67.3% of the cellular volume.
320 This inverted correlation between Small and Large aggregates can be explained by the
321 constant cellular proliferation within the bioreactor, therefore when growing, Small
322 aggregates, finally become Large. This explanation is in agreement with the increase in
323 Medium aggregates at weeks 2 that seems to be “fed” by the growing of Small aggregates.
324 Themselves, Medium aggregates “feed” Large aggregates at week 3 by growth and
325 coalescence. It is worth to note that the cellular distribution profile of cells cultured on
326 spheres is not similar to the one of cells cultured on cubes but is shifted from one week (Fig.
327 4-B). Indeed, week 1 profile of cells cultured on spheres is similar to the week 2 profile of
328 cells cultured on cubes. A similar observation can be made for week 2 profile of cells culture
329 on spheres, which is similar to the week 3 profile of cells cultured on cubes.

330 **Fluid flow and mechanical stimulation**

331 Hydrodynamics within the bioreactors depend on the culture medium flow rate, on its
332 viscosity, and on the construct geometry. From images captured by X-ray μ CT, we obtained
333 the scaffold geometry and the real distribution in time and space of the cells within the
334 scanned volume. We focused on the constructs perfused during 3 weeks. Indeed, the
335 comparative analysis between the wall shear stress distribution and the local presence of cells
336 is statistically more robust when the scaffolds are more cellularized. We calculated fluid flow
337 and wall shear stress on the scaffold surface at the beginning of cultivation (*i.e.* without the
338 cellular phase) using an in-house lattice-Boltzmann code.

339 Fig. 5-A1, 5-B1 and Fig. 5-A2, 5-B2 show typical maps of the fluid velocity modulus as well
340 as a map of the wall shear stress in the same horizontal plane. We note that the highest wall
341 shear stress values arose around the pores where the fluid flows most quickly. We determined
342 the probability density function (PDF) of the shear stress exerted by the fluid on the scaffold

343 surface at the beginning of cultivation. The PDF were computed with a bin size of 0.5 mPa.
344 Fig. 5-A3 and Fig. 5-B3 show that the PDF of the wall shear stress is narrower for the
345 stacking of cubes than for the stacking of spheres and the WSS is half as low on average.
346 Indeed, for the stacking of spheres (resp. cubes), the wall shear stress within the bioreactor
347 ranges between 0 and 130 mPa (resp. between 0 and 100 mPa). The first, the fifth and the
348 ninth deciles are equal to 5 mPa, 18 mPa and 37 mPa (resp. to 2 mPa, 9 mPa and 21 mPa).
349 The mean WSS is equal to 20 mPa (resp. 11 mPa) and the standard deviation of the PDF is
350 equal to 13 mPa (resp. 8 mPa). We emphasize that the error bars (which presently represents
351 one standard deviation bellow and above the mean value, calculated from three independent
352 experiments) on the PDF are larger for the stackings of cubes. This is attributed to the greater
353 variability of stacking.

354 To quantify the wall shear stress occurring in the regions that the cells preferentially cover,
355 we computed the PDF of the wall shear stress at the beginning of cultivation conditioned to
356 the local presence of cells after three weeks of cultivation (Fig. 5-A3 and 5-B3). It appears
357 that the conditional PDF is slightly shifted toward higher shear stresses (WSS mean value
358 increases by 4%) for spheres whereas it is slightly shifted toward lower WSS (WSS mean
359 value decreases by 4%) for cubes. This effect is more significant at low WSS: the first decile
360 is shifted from 5 mPa to 7 mPa for the spheres where as it is shifted from 2 mPa to 1 mPa for
361 the cubes.

362 In order to link the values taken by the wall shear stress to the presence of cells (regardless of
363 the frequency of the WSS values within the bioreactor), we calculated the ratio of the
364 conditional PDF to the “standard” one. This ratio can be interpreted as the occupancy of the
365 scaffold surface sites characterized by a given value of the wall shear stress, normalized by
366 the construct surface coverage with cells. We defined the occupancy of a given class of
367 scaffold sites as the fraction of sites (from this class) covered by cells after three weeks of

368 cultivation. The construct surface coverage is the fraction of scaffold surface covered by cells
369 regardless of the wall shear stress value. Fig. 5-A4 and Fig. 5-B4 present the variations of the
370 PDF ratio over the interval [0 mPa; 60 mPa] for the stacking of spheres and the stacking of
371 cubes, respectively. We chose 60 mPa as an upper bound since it coincides with the 99th
372 centile of the PDF for spheres (the 99th centile of the PDF for cubes is lower, *i.e.* about 40
373 mPa). We adapted the bin size according to the intensity of the WSS to obtain more reliable
374 values: since the probability to find large values of the WSS within the bioreactor is low (the
375 PDF tends to zero as the WSS becomes high), the uncertainty on the PDF values is high in
376 that range and the uncertainty on the PDF ratio even higher.

377 Fig. 5-A4 shows that for the stacking of spheres, the relative occupancy of the surface sites is
378 minimal when WSS is zero. It increases with WSS over the interval [0 mPa ; 20 mPa] and
379 reaches a plateau above 20 mPa. The surface sites with WSS below 10 mPa are less covered
380 by cells than average (PDF ratio lower than 1). The picture is different and less clear for the
381 stacking of cubes (Fig. 5-B4). It seems more difficult to identify a definite trend. The relative
382 occupancy of the surface sites appears maximal when WSS is close to zero. It decreases with
383 WSS over the interval [0 mPa; 10 mPa]. The relative occupancy reaches a plateau between
384 10 mPa and about 20 mPa. Above 20 mPa, the relative occupancy appears erratic and
385 scattered: a reasonable increase in bin size does not compensate for the decrease in the
386 number of events per bin.

387

388 Discussion

389 Methodology combining histological procedure and image analysis allows the description and
390 quantification of cell proliferation within bioreactor⁵³. However, this method can only
391 generate a crude rather than comprehensive depiction of the 3D scaffold, primarily because its
392 destructive nature leads to an incomplete representation of the 3D tissue. Over the last ten
393 years, non-destructive three-dimensional image-based technology has made steady progress
394 (*i.e.* the ability to visualize micrometric objects)⁵⁴. The notion of virtual histology, which
395 involves a combination of tomography imaging and image processing to analyse 3D tissue-
396 engineered emerged over this period^{28,29}. Among these technologies, high-resolution X-ray
397 micro-tomography offers non-invasive 3D visualization of biological scaffolds^{26,27}. To study
398 the effect of WSS on cells, the ideal method is to collect relevant information at the single-cell
399 scale from the macroscopic bioreactor scale. This is made possible by coupling μ CT imaging
400 and numerical simulation. Computational fluid dynamics approaches have shown promising
401 results to estimate wall shear stress distribution in idealized geometries and numerically
402 reconstructed scaffold geometry from X-ray tomography^{33–35,55,56}. To date, this strategy has
403 been implemented with considerable success on single scaffold with a single-cell resolution
404 without contrast agent^{28,29}. This approach enables the assessment of cell proliferation (and
405 mineralization) within the volume of 3D scaffolds. However, this method is difficult to apply
406 at the macroscopic bioreactor scale. Although it is now possible to image with μ CT volumes
407 of several cubic centimeters by helical acquisition with a resolution close to that of a single-
408 cell, the time required for the acquisition (30 hours in our case), the volume of data generated
409 (more than 100 Giga octets in our case), and the computing power needed to visualize the
410 image does not allow for this method to be used routinely. Compromises are necessary in
411 terms of resolution. That is why in this study, despite being aware that we are losing some
412 amount information, we chose a voxel of $17*17*17 \mu\text{m}^3$ equal to at least the volume of a

413 couple of fibroblasts⁵² resulting in an acquisition time of 1.5 hours for 15 Giga octets. Major
414 limitations of μ CT are related to the mechanisms involved in image acquisition. Scanned
415 biological materials become visible based on their attenuation of the X-ray energy beam⁵⁷.
416 Moreover, to be able to visualize the cell phase, we demonstrated the feasibility of improving
417 cell contrast at the macroscopic bioreactor scale by using osmium tetroxide⁵⁸ on constructs.
418 Then, from the μ CT acquisitions, an accurate three-dimensional virtual representation of
419 constructs can be generated. This approach enables the qualitative and semi-quantitative
420 assessment of cell proliferation within the volume of constructs and allows us to generate 3D
421 objects that are directly usable for CFD simulations.

422 The interactions between fluid flow and living media are complex, and far from being fully
423 understood. Cell and tissue metabolism are typically influenced by the transport phenomena
424 of nutrients and oxygen that the flow controls. They may also be directly affected by the
425 viscous shear stresses⁵⁹ that fluid flow produces.

426 We simplified the perfusion culture method that is described in David & al² (stack of
427 microporous scaffolds). Our decision to reduce the double porosity to a single one was
428 motivated by the fact that the impact of WSS on cells has already been described during cell
429 cultures for microporous scaffolds^{29,30} but never in the macroscopic porosity within a packed
430 bed bioreactor.

431 We checked that in our experiments, the transport of glucose and oxygen are not limiting
432 phenomena. An estimate of the relative decrease in concentration in species i between the
433 bioreactor inlet and outlet is given by:

434
$$\frac{\Delta C_i}{C_0^i} = \frac{N_{cells} V_m^i}{Q C_0^i} \quad (9)$$

435 where N_{cells} is the number of cells within the bioreactor at the time considered, V_m^i is the
436 uptake of species i and C_0^i is the concentration in species i at the inlet. The glucose uptake rate

437 is equal to about 10^{-16} mol.cell⁻¹.s⁻¹ ⁶⁰ the oxygen uptake rate to 6×10^{-17} mol.cell⁻¹.s⁻¹ ⁶¹.
438 The glucose initial concentration of the culture medium is equal to $C_0^{gl} = 25$ mol.m⁻³, the
439 oxygen initial concentration to $C_0^{O_2} = 0.21$ mol.m⁻³. The maximum number of cells obtained
440 after 3 weeks of cultivation (among the three experiments) was about 60 million. The relative
441 decrease in glucose concentration between the bioreactor inlet and outlet is then negligible
442 (about 0.1%) and the relative decrease in oxygen concentration is less than 10%.
443 Furthermore, the cells located in the depth of the tissue are well fed. Assuming one-
444 dimensional steady diffusion, the relative decrease in species i across the cell layer growing
445 on the sphere/cube surface reads:

$$446 \quad \frac{\Delta C_i}{C_0^i} = \left(\cosh \left(\sqrt{\frac{V_m^i}{\vartheta \mathcal{D}_i}} s \right) \right)^{-1} \quad (10)$$

447 where s is the cell layer thickness, ϑ the average volume of a fibroblast and \mathcal{D}_i the diffusion
448 coefficient of species i . We measured that the maximal cell layer thickness at week 3 is of the
449 order of 100 μ m. The diffusion coefficient of oxygen is equal to $\mathcal{D}_{O_2} = 3 \times 10^{-9}$ m².s⁻¹ and
450 the diffusion coefficient of glucose to $\mathcal{D}_{gl} = 6.7 \times 10^{-10}$ m².s⁻¹. The relative decrease in
451 glucose concentration across the cell layer is then estimated at 35% and the relative decrease
452 in oxygen concentration at 6%. The oxygen (resp. glucose) concentration within the cell layer
453 remains greater than the Michaelis-Menten constant for oxygen, *i.e.* $K_M^{O_2} = 6 \times 10^{-3}$ mol.m⁻³
454 (resp. glucose, *i.e.* $K_M^{gl} = 9$ mol.m⁻³).

455 It should be noted that the conclusion could be different if the material constituting the beads
456 or the cubes was porous (like coral for example). In that case, the resistance to solute transfer
457 in the micropores of the material should be taken into account.

458 Using a lattice-Boltzmann method, we were able to simulate the fluid flow within our
459 bioreactors and retrieve the local values of WSS on the scaffold' surface at the beginning of
460 cultivation. Two kinds of scaffold were examined, *i.e.* stacking of cubes of 3 mm edge and

461 stacking of spheres of 2 mm diameter. We found that the distributions strongly differ: the
462 PDF is narrower for the stacking of cubes than for the stacking of spheres. Furthermore, the
463 mean WSS is lower for cubes (11 mPa) than for spheres (20 mPa). It is worth noting that the
464 porosity of the stackings were very close, *i.e.* 46% and 45.5%, respectively, and the specific
465 wetted areas were also similar, *i.e.* 707 m⁻¹ and 840 m⁻¹, respectively. We deduce that the
466 discrepancies in WSS distribution are presently mainly due to the shape of the particles
467 forming the granular bed (*i.e.* the scaffold) and to their spatial arrangement. Besides, the
468 geometry of the particles directly impacts the way they pile up (see Fig. 1).

469 The ultimate goal of this study is knowing and controlling the level of stimulation in advance
470 at the interface between the fluid and the cells. During the cell culture, the average WSS
471 depends on the superficial velocity and on the construct porosity within the bioreactor. It is
472 possible that simulations we performed on empty scaffolds would be slightly different after
473 the start of culture ³⁰. The porosity of the construct may also vary over time due to cell
474 proliferation and matrix deposition. In certain experiments conducted on highly porous (80%)
475 PLA-nonwoven-fiber-mesh scaffold, it has been demonstrated ³⁰ that the levels of WSS
476 increase as a function of culture time. However, the pore size within this scaffold (and more
477 generally scaffolds used in bone tissue engineering), around 100-200µm, is very different
478 from that within a stack of cubes/beads in the bioreactor (≈ 1 mm). In both cases, the increase
479 in biomass within pores leads to a decrease in porosity, but pore clogging and subsequent
480 liquid flow redistribution within the bioreactor occur in different time scales. In the present
481 study, because the volume fraction of cells (about 1%) after three weeks of perfusion remains
482 small compared to the porosity of the spheres-based scaffold (about 45%), because the pores
483 within the stacks are macroscopic, we expect that the PDF of the WSS within the construct
484 during the time course of cultivation does not significantly differ from the PDF at the
485 beginning of cultivation.

486 As described in the results section, we also computed the PDF of the wall shear stress at the
487 beginning of cultivation conditioned to the local presence of cells after three weeks of
488 cultivation. We noted that for the same kind of particles, the conditional PDF is similar to the
489 “standard” PDF: the general shape of the conditional PDF is more influenced by the WSS
490 distribution within the bioreactor than by the presence of cells. In order to isolate the relation
491 between the value of the wall shear stress and the presence of cells (regardless of the
492 frequency of that WSS value within the bioreactor), we calculated the ratio of the conditional
493 PDF to the “standard” one. This ratio represent the occupancy of the scaffold surface sites
494 characterized by a given value of the wall shear stress, normalized by the construct surface
495 coverage with cells.

496 We first note that, whatever the particle shape, *i.e.* cube or sphere, the relative occupancy of
497 the surface sites by cells remains high (greater than 0.6) in the WSS range (from 0 mPa up to
498 60 mPa) presently investigated. We deduce that this WSS interval is generally favourable to
499 cell proliferation. This finding is in line with the results reported by the literature for
500 osteocompetent cells. *In vitro*, shear stresses ranging from 1 to 10 mPa enhance the cell
501 proliferation²³, from 1 mPa to 50 mPa improve proliferation, matrix production, and
502 mineralization^{1,12,62,63} and values greater than 600 mPa^{10,12,22,64,65} are detrimental for cell
503 viability and adhesion. However, we hypothesize that with osteoblast cells, a longer culture
504 time would be necessary to obtain the same cell expansion within the bioreactor than what is
505 observed with fibroblasts after three weeks (osteoblasts doubling time is twice that of
506 fibroblasts, about 48 hours against 24). In addition, the choice of fibroblasts rather than
507 osteoblasts may be justified by the fact that our objective was to statistically correlate
508 proliferation with shear levels. With osteoblasts, would have observed both proliferation and
509 differentiation, thereby undermining our analysis.

510 As reported in the results section, we may distinguish different WSS intervals according to the
511 relative occupancy of the scaffold surface sites by cells. These intervals depend on the shape
512 of the particles constituting the granular bed. For the stacking of spheres, the relative
513 occupancy of the surface sites by cells is maximal when WSS is greater than 20 mPa. For the
514 stackings of cubes, the relative occupancy is maximal when the WSS is lower than 10 mPa. In
515 the meantime, the quantification of cell proliferation reveals that the cellular volume at week
516 3 is four times higher in the stacking of spheres than in the stacking of cubes (*i.e.* about 61
517 mm³ against 16 mm³). We expect that the cell proliferation starts from the regions where the
518 wall shear stress is low since cells are initially single (or possibly grouped together into small
519 monolayered patches) at the scaffold surface and proliferation in that configuration requires
520 wall shear stress as low as 5 mPa ¹². The maximal occupancy at zero WSS observed for the
521 stacking of cubes is a direct consequence of that proliferation phase. When the cells form a
522 multi-layered tissue-like structure, higher values of the WSS (up to 50 mPa) become also
523 conducive to cell proliferation (and possibly even more conducive than lower values of WSS).
524 This is precisely what we observe for the stackings of spheres. The formation of multi-layered
525 structure require 3-D environments (at the cell scale). Such sites actually exist in stackings of
526 beads: they are typically located at the contact point between beads ⁵³. On the contrary,
527 stackings of cubes seem to have fewer sites able to induce 3-D (multi-layered) proliferation.
528

529 **Conclusion**

530 Using a lattice-Boltzmann method, we computed the flow within the bioreactor and retrieved
531 the local WSS potentially experienced by the cells during the cultivation time course. At a
532 steady perfusion flow rate, the range of WSS occurring within the construct and influencing
533 cell proliferation strongly depends on the geometry of the granular materials constituting the
534 3D based construct. Depending on the geometry of the scaffold considered, it seems that a
535 given level of shear stress may be more or less beneficial in terms of cell proliferation, this
536 benefit is not universal but seems geometry dependent.

537 This work presents a step toward a better mastering of tissue growth in a perfusion bioreactor.
538 By knowing a priori the optimum level of mechanical stimulation required for cell expansion
539 enhancement, the bioreactor perfusion flow rate can be adjusted in accordance with the
540 optimal range presented in this study to obtain, on average, the desired stress value, within the
541 construct. Also, we anticipate that our results, by reverse engineering, will help to design a
542 bioreactor or scaffolds so that the majority of the shear stress values that the cells will
543 experience during an perfusion culture are included in the ranges described in the present
544 paper as beneficial for cell proliferation.

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550

551 **Author Disclosure Statement**

552 The authors have no conflict of interest to declare, including competing financial interests. All
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555

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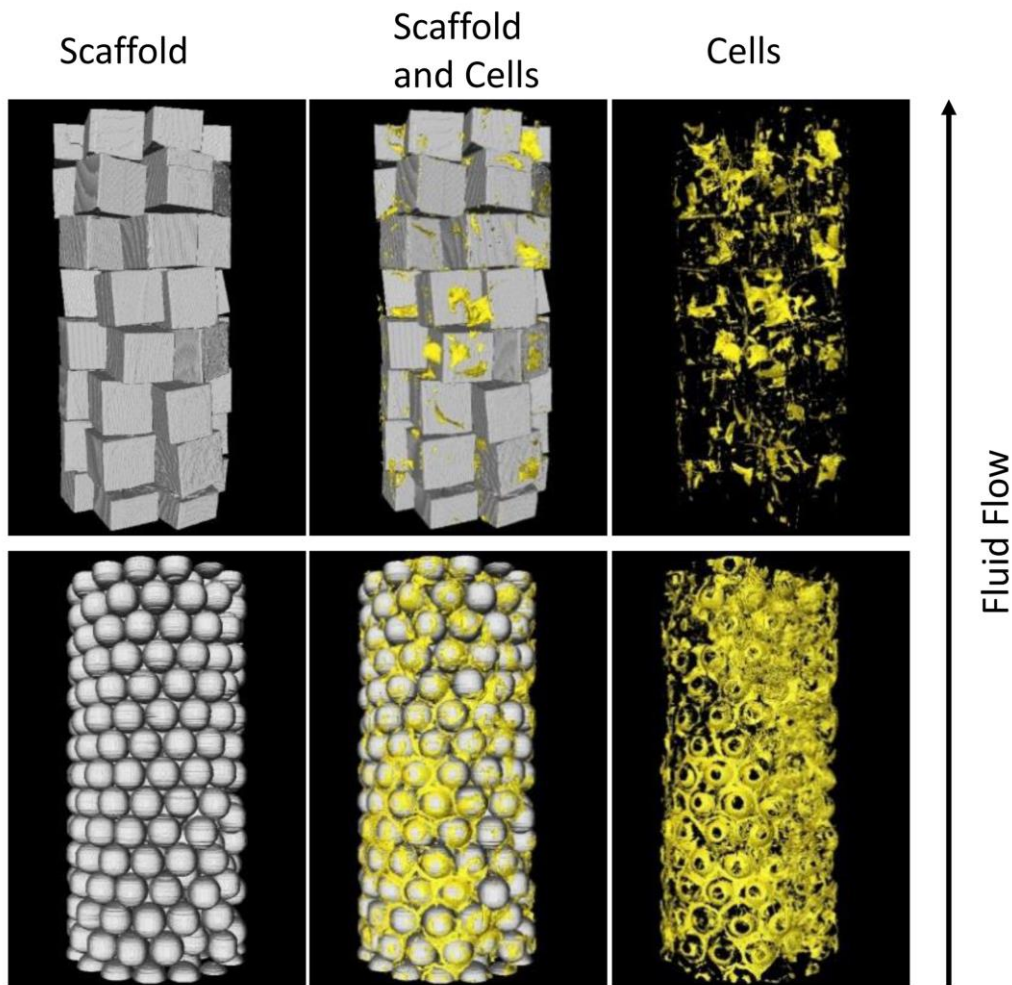
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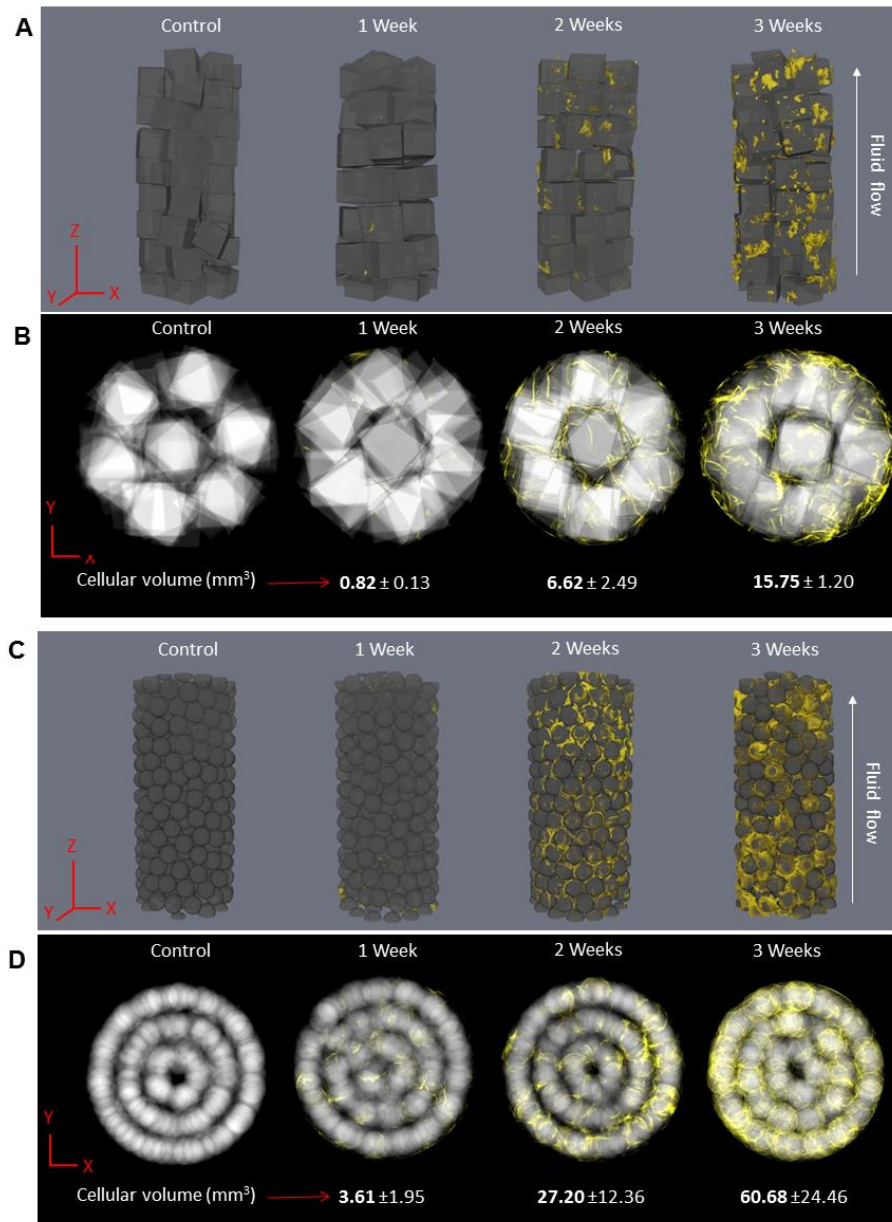
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756 **Figure 1: Cell visualization by X-ray micro Ct within perfusion bioreactors**

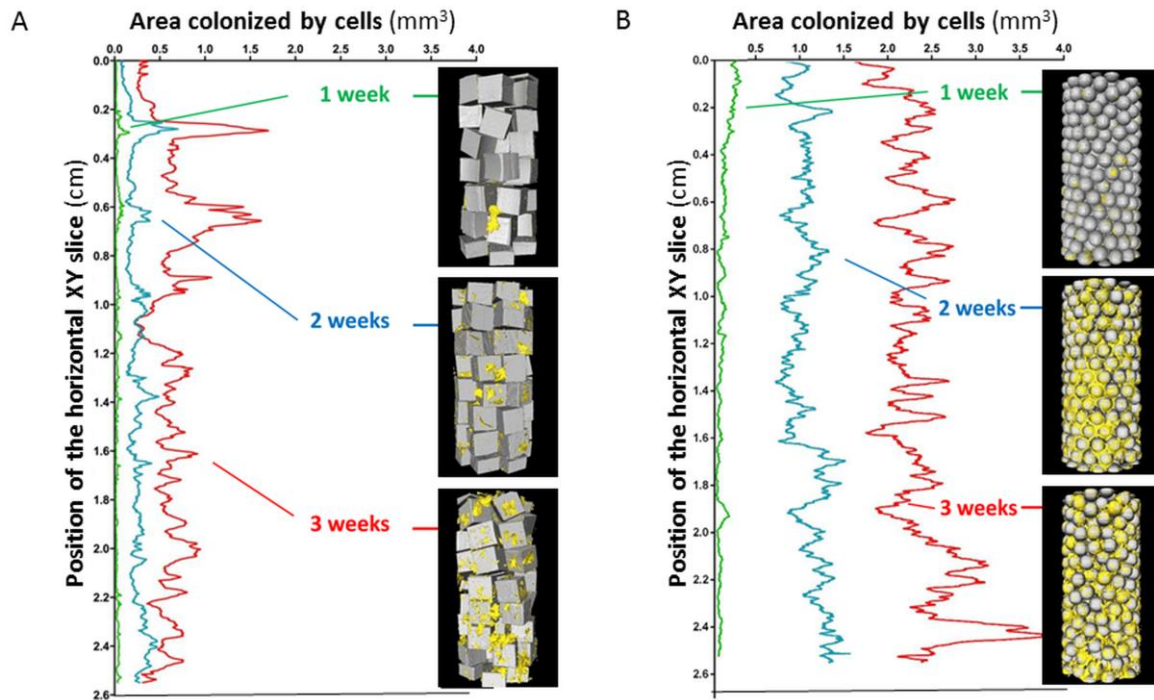
757 3D-reconstruction of X-ray μ CT acquisitions of cell culture in bioreactor enables the
 758 differentiation of scaffold from cells. PDMS cubic scaffold and POM spheres were seeded
 759 with fibroblasts and cultured under an upward flow of 10 mL/min for 3 weeks. Cells were
 760 fixed in 4% paraformaldehyde + 0.5 % glutaraldehyde, stained with osmium tetroxide and
 761 visualized by X-ray μ CT. Based on image contrast enhanced by OsO_4 staining, image
 762 processing prior to 3D-reconstruction uses threshold methods to convert grey-scaled images
 763 into a binary ones. Staining and image binarization allow the mapping of native cellular
 764 organization.



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766 **Figure 2: Cells grow homogeneously over time within perfusion bioreactors**

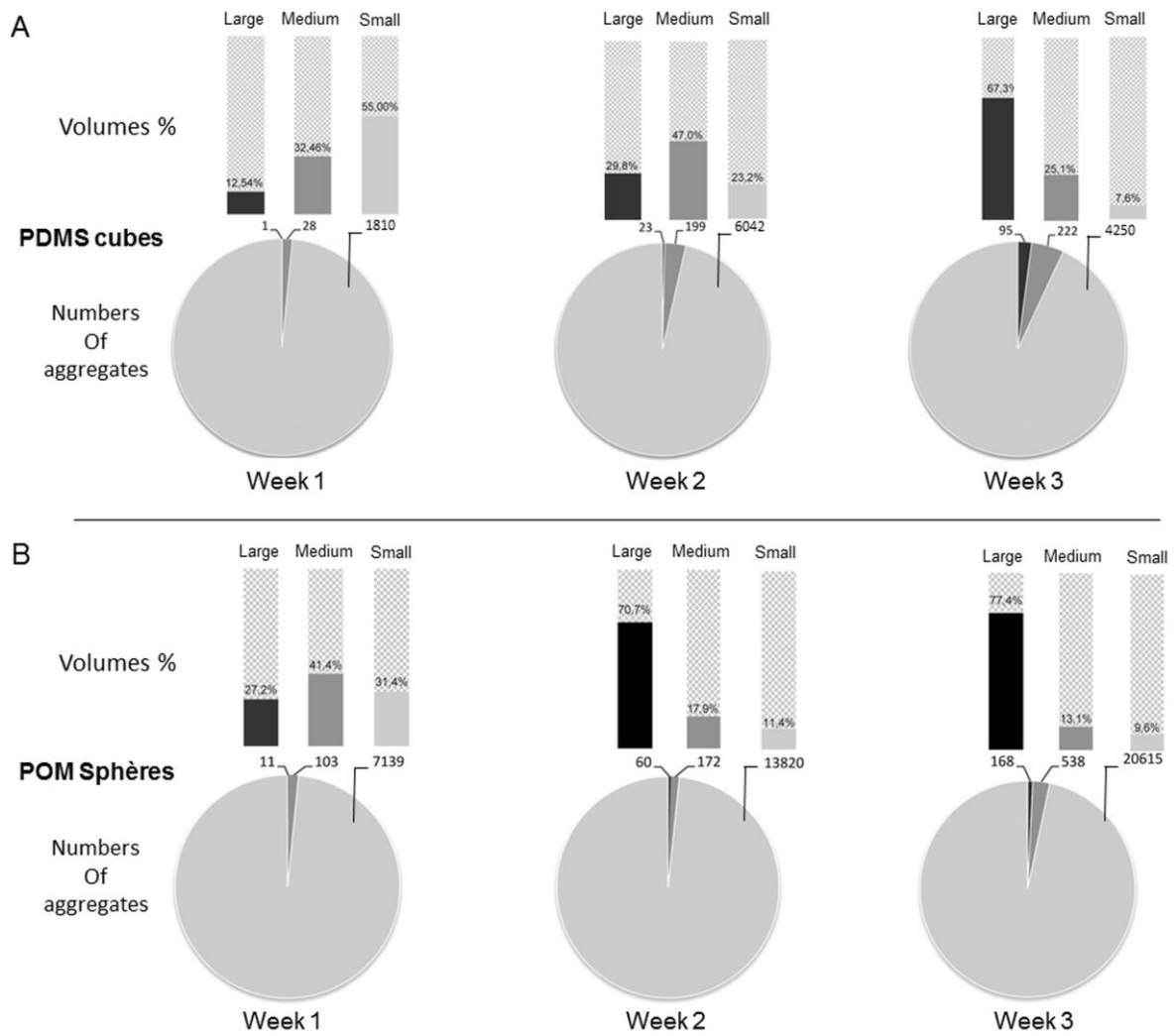
767 3D-reconstructions and Z projections of X-ray μ CT acquisitions of bioreactor cell cultures
 768 over time show a homogeneous cell proliferation. Spheres and cubes-based bioreactors were
 769 seeded with fibroblasts and fixed after 1, 2 or 3 weeks of culture under perfusion flow. Non-
 770 seeded scaffold is shown as control. Both 3D reconstructions and Z projections illustrate the
 771 homogeneous cell growth within the whole volume of the bioreactor regardless the
 772 architecture of the scaffold. The total cellular volume was measured based on 3D-
 773 reconstructions for the 3 time points.



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775 **Figure 3: Quantification of cell proliferation, distribution and organization within**
 776 **perfusion bioreactors over time**

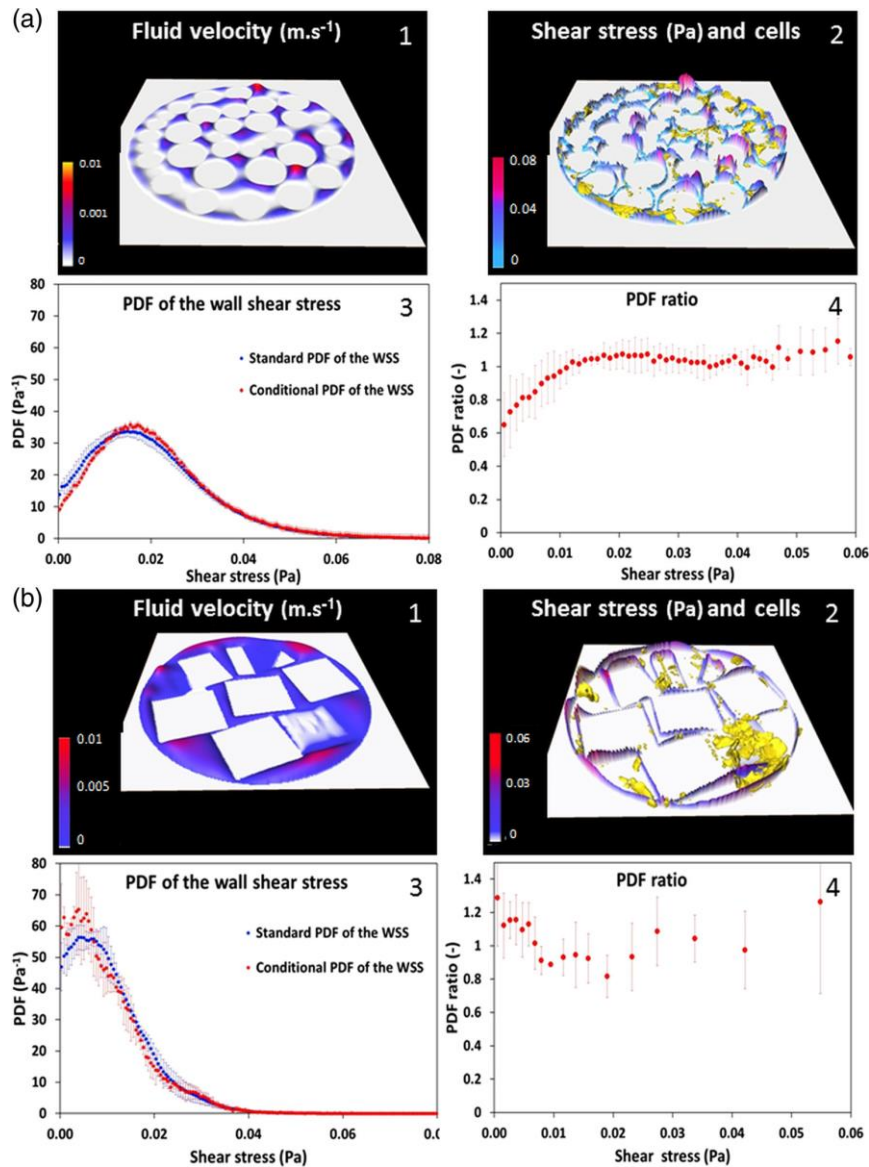
777 Local cell proliferation was measured for each XY section: surface area covered by cells is
 778 plotted as a function of its position along the Z-axis (flow direction), for the 3 time points
 779 (green: week 1, blue: week 2, red: week 3).



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781 **Figure 4: Constant cellular proliferation feeds the pool of large cellular aggregates**

782 Using 3D-reconstructions for the 3 time points, number of cellular aggregates and the
 783 respective volume they occupy within the cellular phase were measured for spheres-based (A)
 784 and cubes-based (B) constructs. For both scaffolds, large cellular aggregates remain the less
 785 numerous though they rapidly represent the more volumic category of cellular aggregates.



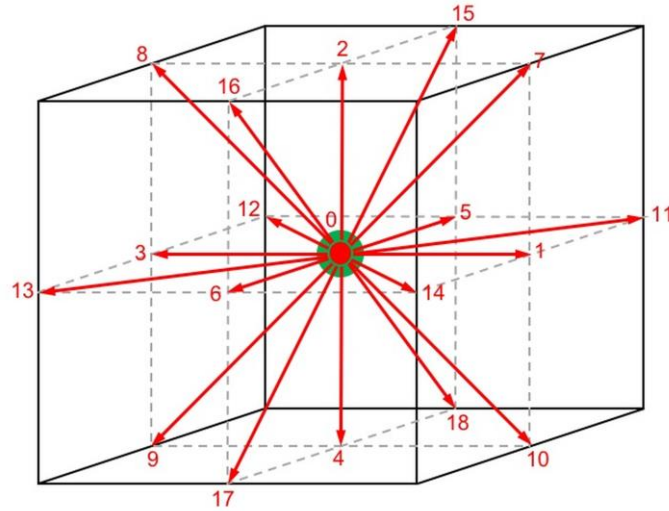
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787 **Figure 5: 3-D lattice-Boltzmann simulations enables fine description of the optimal**
 788 **shear stress for cell proliferation.**

789 Map of the fluid velocity modulus (A1-B1), map of the wall shear stress and spatial
 790 distribution of cells (A2-B2) in the same horizontal plane. Standard wall shear stress
 791 Probability Density Function (PDF) and conditional PDF (*i.e.* the PDF of the wall shear stress
 792 conditioned to the local presence of cells after three weeks of perfusion) are simultaneously
 793 plotted as a function of wall shear stress (A3-B3). The PDF ratio (which represents the
 794 occupancy of the sphere surface sites characterized by a given value of the wall shear stress)
 795 is plotted as a function of the wall shear stress (A4-B4).

796 **Figure S1: Particle velocity vectors associated with the $d3q19$ lattice-Boltzmann**
797 **method**

798 Voxel (black solid line), lattice node (in green) and particle velocity vectors (in red).



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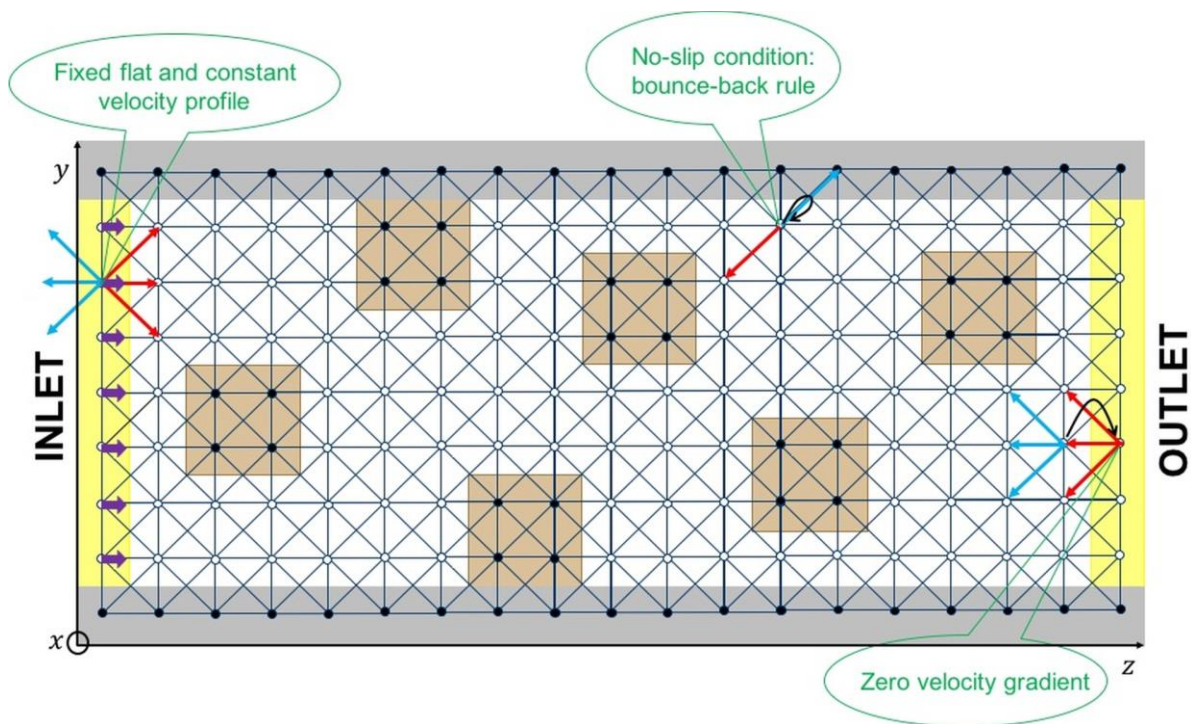
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815 **Figure S2: Schematic of the computational domain**

816 The walls of the bioreactor are displayed in grey and the scaffold in brown. “Fluid” nodes are
817 represented by small hollow circles, “solid” nodes by black filled circles and the links
818 between nodes by black solid lines. Yellow zones correspond to the inlet and to the outlet. At
819 the inlet, the particle populations entering the domain (associated with the red vectors) are
820 adjusted with respect to the particle populations leaving the domain (blue vectors) in order to
821 achieved the set fluid velocity profile (purple arrows). At the outlet, the particle populations
822 entering the domain are equal to the corresponding populations at the penultimate nodes (with
823 respect to the direction of the flow) in order to achieve the zero velocity gradient ⁴⁹.
824 When a particle population encounters an obstacle, it reverses its direction (bounce-back
825 rule).



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